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Citation for published version:

Fernandez-Tornero, C, Boettcher, B, Rashid, UJ & Mueller, CW 2011, 'Analyzing RNA polymerase III by electron cryomicroscopy' *Rna biology*, vol 8, no. 5, pp. 760-765., 10.4161/rna.8.5.16021

Digital Object Identifier (DOI):

[10.4161/rna.8.5.16021](https://doi.org/10.4161/rna.8.5.16021)

Link:

[Link to publication record in Edinburgh Research Explorer](#)

Document Version:

Author final version (often known as postprint)

Published In:

Rna biology

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RNA Biol. 2011 Sep-Oct; 8(5): 760–765.

PMCID: PMC3256353

Published online 2011 September 1. doi: [10.4161/rna.8.5.16021](https://doi.org/10.4161/rna.8.5.16021)

Analyzing RNA polymerase III by electron cryomicroscopy

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Received March 1, 2011; Revised April 28, 2011; Accepted May 14, 2011.

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Abstract

Recent electron cryomicroscopy reconstructions have provided new insights into the overall organization of yeast RNA polymerase (Pol) III, responsible for the synthesis of small, non-translated RNAs. The structure of the free Pol III enzyme at 10 Å resolution provides an accurate framework to better understand its overall architecture and the structural organization and functional role of two Pol III-specific subcomplexes. Cryo-EM structures of elongating Pol III bound to DNA/RNA scaffolds show the rearrangement of the Pol III-specific subcomplexes that enclose incoming DNA. In one reconstruction downstream DNA and newly transcribed RNA can be followed over considerably longer distances as in the crystal structure of elongating Pol II. The Pol III transcription machinery is increasingly recognized as a possible target for cancer therapy. The recent cryo-EM reconstructions contribute to the molecular understanding of Pol III transcription as a prerequisite for targeting its components.

Key words: transcriptional regulation, RNA polymerase III, electron cryomicroscopy, transcription elongation, transcription initiation, TFIIB, Maf1

Introduction

While in bacteria and archaea one single RNA polymerase (Pol) transcribes the entire genome, eukaryotic cells use three distinct enzymes each specialized in the transcription of specific classes of genes. Pol I synthesizes a large pre-rRNA that is later processed giving rise to 28S, 18S and 5.8S RNAs. Pol II produces all mRNA and most snRNA and microRNA. Finally, Pol III synthesizes a set of small, non-translated RNAs involved in the regulation of essential cellular processes such as protein synthesis, RNA processing and protein transport.¹ These non-translated RNAs include all tRNAs, 5S rRNA, splicing U6 RNA, signal recognition particle 7SL RNA, RNA components of RNase P and RNase MRP, and rRNA methylating snR52.

Pol III consists of 17 different subunits with an overall mass of 0.7 MDa and is the largest and most complex among the three eukaryotic RNA polymerases. A tensubunit horse-shoe shaped core contains the two largest Pol III subunits (C160 and C128) forming the active centre and the nucleic acid binding cleft, two assembly subunits shared with Pol I (AC40 and AC19), five peripheral subunits shared with Pol I and II (ABC27, ABC23, ABC14.5, ABC10β and ABC10α), and C11, a subunit that participates in RNA cleavage during backtracking.² Attached to the core, a subcomplex comprising subunits C17 and C25 forms an elongated stalk that provides a platform for initiation factors and is also involved in the interaction with newly synthesized RNA.^{3,4} The stalk completes the group of 12 subunits that have counterparts in all three eukaryotic RNA polymerases. In addition, Pol III contains five specific subunits (C31, C34, C37, C53 and C82) organized in two distinct subcomplexes. One subcomplex corresponds to the C37/C53 heterodimer, which is the counterpart of Pol I subunits A49/A34.5 and is distantly related to Pol II initiation factor TFIIF.^{5,6} The C37/C53 heterodimer interacts with DNA inside the DNA-

binding cleft and is involved in transcription initiation, elongation, termination and reinitiation.^{7,8} The other subcomplex corresponds to the C31/C82/C34 heterotrimer, which shows weak homology to the Pol II general transcription factor (TF) IIE and is involved in transcription initiation.^{5,9} Subunit C34 is predicted to contain three tandem winged-helix (WH) domains. It interacts with subunit Brf1, part of the Pol III-specific general transcription factor TFIIB bound to promoter DNA, and is essential for open complex formation.^{10,11}

Electron cryomicroscopy (cryo-EM) provided the first structural information of the complete Pol III enzyme at 17 Å resolution.¹² The crystal structure of the C17/C25 stalk subcomplex⁴ and recently the structure of the Pol III specific subunit hRPC62,¹³ the human ortholog of *Saccharomyces cerevisiae* C82, have also become available. Two recent papers now describe improved cryo-EM reconstructions of Pol III alone and in complex with nucleic acids or the Maf1 transcriptional repressor,^{14,15} shedding light on fundamental aspects of Pol III mediated RNA synthesis. Here, we will describe the most important features of this enzyme and discuss the current understanding of the structural biology of Pol III transcription.

RNA Polymerase III at 10 Å Resolution

The most detailed cryo-EM reconstruction to date has been determined to a resolution of 9.9 Å,¹⁴ which allowed the identification of isolated α -helices in the Pol III core. This level of detail was possible due to a combination of careful biochemistry, massive data collection of more than 100,000 particles and sorting of different stoichiometric and conformational states inside the data set. The resulting structure allows the precise description of the enzyme architecture and also provides insight into the conformational dynamics of Pol III.

Overall, Pol III resembles the other two eukaryotic RNA polymerases in its core region and it also shows an elongated stalk that is however bent away from the clamp element formed by subunit C160. Fitting the crystal structure of Pol II in a stepwise manner (first the core, then the stalk) into the cryo-EM reconstruction of Pol III suggests that all core and stalk subunits are located at similar positions in both enzymes (Fig. 1). The only ambiguity concerns subunit C11 as its corresponding Pol II subunit Rpb9, partly lies outside the Pol III EM envelope. Whereas in Pol II RNA cleavage during backtracking requires the presence of TFIIS next to the active site, in Pol III this function has been assigned to subunit C11,² and it is therefore conceivable that in Pol III subunit C11 occupies a different position close to the active site.

In the EM reconstruction of Pol III its two specific subcomplexes are well-defined, thus allowing a first attempt to unveil their internal organization and the way they act to modulate Pol III mediated transcription.¹⁴ Each subcomplex binds to one side of the cleft towards its downstream end, making the DNA-binding tunnel longer than in Pol II. The C37/C53 heterodimer binds on the lobe of subunit C128 at one side of the cleft, whereas the C31/C82/C34 heterotrimer attaches to the clamp of subunit C160 on the opposite side of the cleft and establishes connections to the stalk. It is the presence of the heterotrimer that probably causes the stalk to bend away from the clamp and towards the saddle element in subunit C160.

Positioning of Pol III Subcomplexes into the Cryo-EM Reconstructions

Figure 1 shows the positioning of the Pol III subcomplexes into the EM reconstruction of Fernandez-Tornero and co-workers.¹⁴ The C37/C53 subcomplex is held together by a dimerization module that comprises one third of its total mass. This module is similar to an equivalent dimerization module present in Pol I subcomplex A49/A34.5 and in TFIIF.^{5,16} Its position next to the lobe element of subunit C128 has been determined by antibody labeling followed by EM visualization,¹⁴ and is coherent with the position of TFIIF on Pol II determined by cross-linking experiments,¹⁷ as well as with that of subcomplex A49/A34.5 in Pol I.⁶ Native mass spectroscopy demonstrated that the C37/C53 heterodimer is relatively loosely connected to Pol III and easily dissociates from the core.^{18,19} The remaining domains in subunits C37 and C53 likely extend along the Pol III surface surrounding the lobe, where additional density is observed. They probably also partly occupy a density next to the protrusion of subunit C128, which cannot be fully explained by differences between Pol II and Pol III. From these location subunits C37 and C53 could reach the DNA-binding cleft, consistent with the function of the

C37/C53 heterodimer in transcription initiation, elongation, termination and reinitiation.

The C31/C82/C34 subcomplex has been predicted to be the counterpart of TFIIE.⁵ It binds to the Pol III clamp at the same region where TFIIE attaches to Pol II.¹⁷ Subunit C31 shows no structural similarity to other proteins and contains few secondary structure elements. The analysis of mutations affecting Pol III subunits C31 and C160 suggests that C31 is the principal subunit holding the heterotrimer to the enzyme core and that it locates next to the base of the stalk.^{9,11} The position of subunit C82 on the clamp head, close to subunit ABC27, is equivalent in both recent EM reconstructions.^{14,15} In contrast, the location of C34 differs, in particular regarding the position of its third WH domain, which was placed on top of the clamp¹⁴ or close to the wall element.¹⁵ Whereas the latter position (observed only when Pol III is bound to a DNA/RNA scaffold) is coherent with C34/DNA crosslinking experiments,²⁰ it implies extensive interactions with the two largest Pol III subunits and maybe also with subunit C31. In contrast, mass spectrometry^{18,19} suggests that C34 is the most labile subunit in the whole Pol III complex and has established a linear arrangement for the C31/C82/C34 subcomplex with no direct interactions between C34 and C31.

The recent crystal structure of hRPC62, the human ortholog of yeast C82, combined with biochemical interaction studies provides additional insights into the overall organisation of the C31/C82/C34 subcomplex.¹³ hRPC62 contains four extended winged-helix domains (eWH1 to eWH4) arranged around an α -helical, coiled-coil stalk, whereby domain eWH3 contacts subunit hRPC32 (yeast C31). The authors also observe single- and double-stranded DNA-binding activity of subunits hRPC62 (yeast C82) and hRPC39 (yeast C34), respectively.¹³ Subunit hRPC62 can be positioned in the EM envelope in different possible orientations. In one orientation, eWH1 and eWH4 are solvent exposed to allow interactions with single-stranded DNA.¹³ An alternative orientation as depicted in [Figure 1](#) places eWH4 closer to the stalk, but allows more space to accommodate subunit C34. Although the recent EM reconstructions^{14,15} and the newly available hRPC62 structure¹³ allow better positioning the Pol III-specific subunits, additional structural and biochemical constraints are still required to resolve the remaining ambiguities.

Initiating RNA Polymerase III and its Repression

Regardless of their specific promoter structure, initiation of transcription in all Pol III genes reaches a stage in which TFIIIB, composed by subunits Brf1, Bdp1 and TBP, is bound to upstream promoter DNA.²¹ From this position, TFIIIB recruits Pol III to the transcription start site, forming the closed pre-initiation complex (PIC). Crystal structures of the Brf1-homology region II in complex with TBP and DNA,²² and of TFIIIB (equivalent to the N-terminal half of Brf1) in complex with Pol II^{23,24} and bound to TBP and DNA,²⁵ allow the construction of a closed PIC model. In this model the N-terminal Zn-ribbon of Brf1 rests on the Pol III saddle, next to the stalk base, and accesses the active site, whereas the two cyclin domains are positioned on top of the wall, together with TBP and promoter DNA ([Fig. 2](#)). The Brf1-homology region II of Brf1, attached to TBP, lies at a distance of about 30 Å from C34 that can be easily bridged by the disordered C-terminal tail of Brf1 in agreement with the reported interaction.²⁶

In the closed PIC ([Fig. 2](#), Step 1), the DNA downstream of the TATA box lies above the cleft in close proximity to C34 and the Pol III protrusion, formed by subunit C128 and putatively by extended domains of the C37/C53 subcomplex. From this position subunit C34, in coordination with TFIIIB and domains of subcomplex C37/C53, is able to melt the DNA and proceed to open the PIC ([Fig. 2](#), Step 2). In this process, which in contrast to Pol II does not require energy, WH domains in C34 may play a key role, as observed for other WH-containing proteins.²⁷ Important conformational changes are likely to occur in order to allow the melted DNA to access its binding cleft. Addition of the first RNA nucleotides occurs at this step, but further structural rearrangements of Pol III and the DNA template are required to detach the enzyme from TFIIIB, marking the beginning of the elongation phase.

Maf1, a repressor of Pol III transcription during stress conditions, can block the recruitment of Pol III by promoterbound TFIIIB ([Fig. 2](#), Step 1, dark red). Under normal conditions Maf1 is phosphorylated and resides in the cytoplasm. Following stress signals Maf1 becomes dephosphorylated and imported into the nucleus.^{28,29} The cryo-EM reconstruction of Pol III in complex with Maf1 shows that it binds on top of the clamp element establishing direct interactions with subunit C160 and possibly with subunits C34 and C82.¹⁵ Binding of Maf1 apparently induces a conformational change that mainly affects the Pol III

C31/C82/C34 heterotrimer and prevents the interaction of Pol III with TFIIIB subunit Brf1, and therefore the formation of the closed PIC.

Elongating RNA Polymerase III

The two cryo-EM reconstructions of elongating Pol III use different DNA-RNA scaffolds. One reconstruction at 19 Å resolution uses a minimal scaffold containing a DNA/RNA heteroduplex, short single-stranded RNA and downstream DNA duplex regions, but is lacking single-stranded DNA from the bubble region and the upstream DNA duplex.¹⁵ The other reconstruction at 16.5 Å resolution contains all the above missing regions and therefore better mimics the transcription bubble bound to Pol III during elongation.¹⁴

In this reconstruction a major part of the DNA/RNA scaffold interacts with the DNA-binding cleft, in almost identical positions as in Pol II.³⁰ Nevertheless, a longer stretch of downstream DNA comprising seven additional base pairs (from position +10 to +16) can be seen in the Pol III elongation complex, probably due to the presence of the Pol III specific subcomplexes that elongate the cleft in the downstream direction. Moreover, a new piece of density connecting the DNA/RNA heteroduplex and the stalk appears on top of the saddle element.¹⁴ This density likely corresponds to the complete RNA strand present in the scaffold and can be followed 10 nucleotides further than in the published Pol II elongation complex structure (Fig. 2, Step 3).³⁰ The upstream DNA duplex may occupy a piece of density above the cleft, next to the lobe element, although more data are required to prove this hypothesis (Fig. 2, central inset). Modeling of an ideal B-form DNA into the proposed density suggests that, once detached from TFIIIB, the overall DNA molecule may have a U-shape (Fig. 2, Step 3) instead of the previously proposed L-shape.³¹ A possible U-shaped DNA conformation has not yet been observed in other RNA polymerases and might be specific for Pol III.

Binding of the transcription bubble induces a number of conformational changes in the enzyme, mainly affecting both Pol III specific subcomplexes and the stalk. The stalk moves towards the exiting RNA, whereas both subcomplexes reorganize and move towards the DNA in the cleft. The strongest rearrangement involves subcomplex C31/C82/C34 that moves away from the stalk and towards the cleft and subunit ACB27, which forms one of the polymerase jaws. Interestingly, this makes the heterotrimer more prone to detach from the core as observed by mass spectrometry.¹⁸ On the opposite side of the DNA-binding cleft the region around the lobe and protrusion elements, where subcomplex C37/C53 is attached, also moves towards the cleft. All these changes produce a better grip of Pol III on the DNA, and therefore are believed to enhance the processivity of the enzyme.

Terminating and Re-Initiating RNA Polymerase III

Pol III is mechanistically unique in requiring only a stretch of five thymidine residues on the non-template strand for efficient termination without additional factors.⁸ In the structure of the Pol III elongation complex, an elongated additional density connects the C37/C53 dimerization module with downstream DNA, and this density has been proposed to harbor disordered regions of subunit C53 that become ordered when the transcription bubble has formed.¹⁴ As this position corresponds to the most downstream point of the elongation complex and given the role of the C37/C53 subcomplex in termination,⁸ the observed density could be crucial for the recognition of the Pol III terminator sequence before it reaches the cleft. As proposed, this could slow down elongation and allow conformational changes that lead to Pol III detachment from the DNA template and the RNA product (Fig. 2, Step 4).

Pol III directs several rounds of transcription on the same gene once attached to DNA, an event known as facilitated reinitiation.³² Conformational changes have been postulated to occur, probably affecting the heterotrimer and the stalk, which allow a new recruitment of Pol III to the start site by promoter-bound TFIIIB. The short length of most Pol III genes minimizes the distance between the enzyme and its specific transcription initiation factors (Fig. 2, Step 4). The decrease in the elongation speed due to terminator recognition and also the tight DNA enclosure that we observe in the Pol III elongation complex, likely facilitates reinitiation.

RNA Polymerase III Misregulation and Cancer

Pols I and III are central cellular enzymes that synthesize the RNA components required for protein

synthesis in eukaryotic cells. Together, they contribute up to 80% to the total transcriptional activity in growing cells and therefore need to be tightly regulated.³³ Consequently, in humans upregulation of Pol III transcription and overexpression of Pol III products are often associated with transformed cells and carcinomas (reviewed in ref. ³⁴). Several oncogenes also act directly on Pol III components as described for the oncogene c-Myc that directly binds to TBP and Brf1.³⁵ Similarly, Erk kinase activated by Ras and Raf signaling can phosphorylate TFIIIB and increase its activity.

Otherwise, a number of tumour suppressors including PTEN (phosphatase and tensin homolog), p53 and RB downregulate the activity of Pol III components. p53 and RB act by directly binding to TFIIIB and thereby preventing it to recruit Pol III. As described above Maf1 appears to function similarly by binding to Pol III and inhibiting the interactions with the TFIIIB component Brf1.¹⁵

The increasing number of cases where deregulation of Pol III has been associated with different types of cancer has identified the Pol III transcription machinery as potential target for cancer treatment. A detailed molecular understanding of the overall architecture of Pol III, its general transcription factors and the regulatory mechanisms that control Pol III is required to move further in this direction. Structural insight as provided by the recent cryo-EM reconstructions^{14,15} will contribute directing further efforts.

Acknowledgments

The authors like to thank Sebastian Fribourg for making results available prior to publication and for comments on the manuscript.

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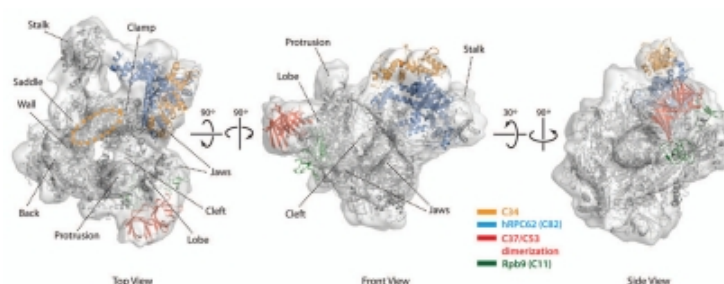
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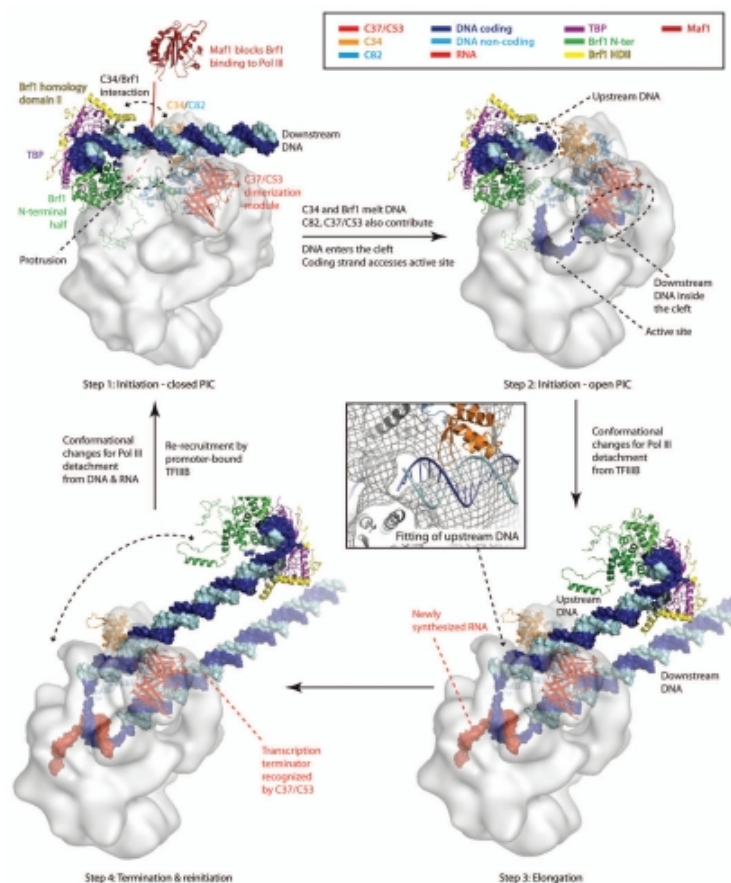
Figures and Tables

Figure 1



Structural organization of the Pol III-specific subcomplexes. The complete 12-subunit Pol II structure (grey) was fitted into the EM density. For the C31–C82–C34 heterotrimer the crystal structure of hRPC62 ¹³ (blue) and a homology model of C34 (orange) were positioned in the cryo-EM map of Pol III (EMDB code 1802). Subunit C31 connects the stalk with subunit C82, but is not depicted in the Figure. An alternative position of subunit C34 as suggested by Vannini and colleagues¹⁵ is indicated by an orange oval. The C37/C53 dimerization domain (red) was manually fitted into the EM density at the opposite side of the DNA cleft. Pol II subunit Rpb9 corresponding to Pol III subunit C11 is depicted in green. The side view corresponds to the orientation shown in [Figure 2](#).

Figure 2



Steps in Pol III transcription. The closed PIC model (Step 1) was obtained by sequential fitting of the Pol II-TF IIB crystal structure (PDB code 3K7A) into the cryo-EM map of Pol III (EMDB code 1802), superposition of the TF IIB-TBP-DNA crystal structure (PDB code 1VOL) onto the TF IIB N-terminal cyclin domain, and superposition of Brf1 homology region II in complex with TBP and DNA (PDB code 1NGM) onto TBP/DNA. An ideal B-DNA was used to extend from the crystal structure. The putative positions of C37/C53, C34 and C82 are shown. The open PIC model (Step 2) was generated as before but, instead of downstream DNA, we fitted the Pol II elongation complex (PDB code 1I6H) into the cryo-EM map of Pol III (EMDB code 1803) and removed the RNA strand. In the elongation and termination models (Steps 3 and 4) the upstream DNA was fitted into an empty density above the cleft (Inset) and elongated with an ideal B-DNA attached to TF IIB/TBP/Brf1.

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